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Additional information on DNA barcoding of the African armyworm, *Spodoptera exempta* (Walker) (Lepidoptera, Noctuidae) from Japan

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Abstract After the occurrence of *Spodoptera exempta* (Walker) as a pest in Japan in 2010, we identified this pest again in the Nansei Is., Japan in 2012 and 2013. Using the method of standard DNA barcoding based on the additional specimens of *S. exempta*, we have identified three haplotypes from Japan. When compared the haplotypes of individuals from Africa, Australia, Papua New Guinea and Japan, the genetic structure of the Asia-Oceanian population was distinguished from that of the African population.

Key words haplotype, Kagoshima, Nansei Is., Okinawa, pest.

Introduction

The genus *Spodoptera* Guenée comprises 30 species, about half of which have been considered as pests of numerous vegetables, grain crops, and pasture. The genus is mainly tropical and subtropical throughout the world with some species migrating into more temperate regions throughout the growing season (Pogue, 2002). According to chronology estimates using four mitochondrial and two nuclear genes by Kergoat *et al.* (2012), the genus *Spodoptera* started its diversification in the Miocene, when open habitats were dominant and were filled with plants belonging to the modern herbaceous angiosperm group.

The African armyworm, *Spodoptera exempta* (Walker) (Fig. 1) is a notorious pest of crops and pasture in Africa and is also distributed from South East Asia and Australia to some Pacific Islands including Hawaii. There is abundant field and laboratory evidence that the moths are capable of migratory flights covering hundreds of kilometers (Gatehouse, 1986). Although several adult specimens had been collected, no damage by this pest species had been reported from Japan before 2010. However, Yoshimatsu *et al.* (2011) for the first time recorded severe damages to graminaceous forage crops, sugar cane etc. by this pest from August to October, 2010 in the Nansei Is., Japan. Uesato *et al.* (2011) also reported details of an outbreak in the Ryukyu Is. at the same time with very impressive illustrations of huge numbers of larvae crossing a road neighboring a pasture area.

Later, the first author (Yoshimatsu) had an opportunity to examine two male *Spodoptera* specimens collected by T. Fukuda from Mt. Shibi-san, alt. 1,067m, Kagoshima Prefecture on 12 August, 2010 and identified them as *S. exempta* (Fukuda, 2012). Fukuda (2012) also wrote that many individuals of the same species seemed to be flying during that night; however, he had collected only the above-mentioned two specimens. He also mentioned that many adult moths were in fact flying on the mainland of Kyushu at almost the same time as severe damages to crops occurred in August, 2010 in Okinawa Prefecture.

As mentioned by Watabiki *et al.* (2013), a survey using a

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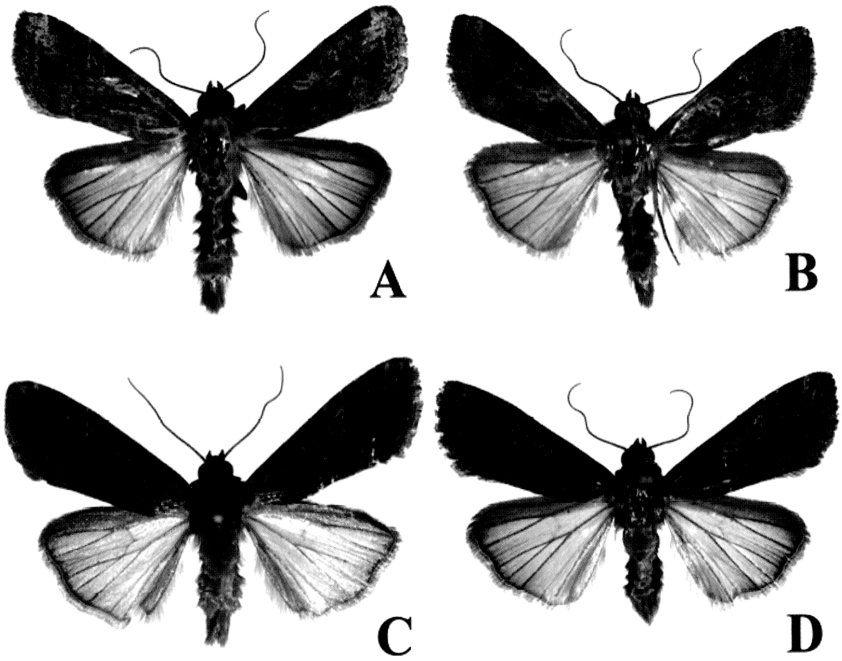

Fig. 1. Adults of *Spodoptera exempta* emerged from the larvae and pupae collected in Kagoshima Prefecture, Japan, 2013. A-B: males, C-D: females.

synthetic sex pheromone for *S. exempta* was conducted in 2010 by the Okinawa Prefectural Plant Protection Center and the Kagoshima Prefectural Institute for Agricultural Development. As a result, several species of *Spodoptera* were collected by the pheromone trap set for *S. exempta* including *S. exempta* itself. As it was difficult to distinguish *S. exempta* accurately from other congeners, they constructed a key to distinguish all eight Japanese species of *Spodoptera* using mainly the male genitalia. In addition, they distinguished six Japanese *Spodoptera* pest species using standard DNA barcoding, in which they recognized two haplotypes of *S. exempta* from Japan.

After the occurrence of *Spodoptera exempta* (Walker) as a pest in Japan in 2010, we identified this pest again in the Nansei Is., Japan in 2012 and 2013. In this paper, we report the results of a survey of the DNA barcoding on the basis of the additional specimens with illustrations of the emerged fresh male and female adults. In particular, this time we for the first time compared the DNA barcoding data from Japan with data from Australia and Papua New Guinea.

Materials and methods

The collecting data of eight male adults which were attracted by the pheromone trap for *S. exempta* from Okinawa Prefecture in 2012 and were subsequently examined are given in Table 1. That relating to 31 adults which were reared from larvae or pupae from Kagoshima Prefecture in 2013 and used are shown in Table 2 and two pairs of them illustrated in Fig. 1. Male and female are distinguished in Fig. 1 and Tables 1 & 2, mainly based on the whiter forewing maculation in the male than in the female and the black scales on the 8th abdominal segment which are lacking in the male as mentioned by Pogue (2002).

DNA was extracted from one leg each of the above-mentioned adults, randomly selected, using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and the leg was homogenized in 200 µl ATL-Buffer with 20 µl proteinase K and incubated at 55°C for over one hour. A DNA fragment of the mitochondrial cytochrome oxidase subunit I (*COI*) gene was amplified using the polymerase chain reaction (PCR) with the standard barcoding primers LCO1490 and HCO2198 (Folmer *et al.*, 1994; Hebert *et al.*, 2003). PCR cycling condition were 94°C for 5 min, 35 cycles of 94°C for 30 s, 47°C for 30 s and 72°C for 1 min with final extension at 72.0°C for 5 min. PCR was performed in a reaction volume of 40 µl using 3.2 µl of each primer (10 µmol/l), 20 µl of GoTaq (Promega), 1.6 µl of template DNA and 12 µl of SDW. We asked Takara Bio Inc. to analyze the nucleotide sequences by direct sequencing using a Big Dye Terminator v 3.1

Table. 1. Collecting information of *Spodoptera exempta* attracted by pheromone traps for *S. exempta* in Okinawa Prefecture, Japan, 2012 and accession numbers. Black accession numbers represent the haplotype of Asia-Oceania 1, red number means that of Asia-Oceania 2 and blue one does that of Asia-Oceania 3, respectively.

No. of individuals with sex	Collecting localities	Collecting date	Names of collecters	Accession numbers
2♂	Tarama-jima Is.	27. ix. 2012	E. Iyoshi	AB915798, AB915799
3♂	Tarama-jima Is.	26. xi. 2012	E. Iyoshi	AB915800～AB915802
2♂	Iriomote-jima Is.	25. ix. 2012	Y. Tomimoto	AB915803, AB915804
1♂	Iriomote-jima Is.	7. xi. 2012	Y. Tomimoto	AB915805

Table. 2. Collecting information of *Spodoptera exempta* captured in Kagoshima Prefecture, Japan, 2013 and accession numbers. Black accession numbers represent the haplotype of Asia-Oceania 1 and red numbers mean that of Asia-Oceania 2.

No. of individuals with sexes	Collecting localities	Up: Collecting date of larvae and/or pupae Down: Date of adults emerged	Names of collectors	Host plants	Accession numbers
1♂4♀	Nishinoomote-shi, Tanegashima Is.	9. viii. 2013 mid. August, 2013	T. Nishioka	Rhodes grass	♂(AB915883) ♀(AB915884, AB915885, AB915886, AB915887)
1♀	Nishinoomote-shi, Tanegashima Is.	16. viii. 2013 23-25. viii. 2013	T. Nishioka	Rhodes grass	AB915888
10♂3♀	Naze, Amami-shi, Amamioshima Is.	21. viii. 2013 30.viii .- 5. ix. 2013	H. Nakamura	Rhodes grass or Sugar cane	♂(AB915889～ABAB915895, AB915896～AB915898) ♀(AB915899, AB915900, AB915901)
2♀	Naze, Amami-shi, Amamioshima Is.	21. viii. 2013 30.viii .- 5. ix. 2013	H. Nakamura	Asparagus	AB915902, AB915903
1♀	Sumiyo-cho, Amami-shi, Amamioshima Is.	21. viii. 2013 30.viii .- 3. ix. 2013	H. Nakamura	Rhodes grass	AB915904
5♂2♀	Setouchi-cho, Amamioshima Is.	21. viii. 2013 30.viii .- 3. ix. 2013	H. Nakamura	Rhodes grass	♂(AB915905～AB915909) ♀(AB915910, AB915911)
2♀	Tokunoshima-cho, Tokunoshima Is.	22. viii. 2013 4-5. ix. 2013	H. Nakamura	Rhodes grass	AB915912, AB915913

Cycle sequencing kit (Applied Biosystems) and an ABI 3730-XL genetic analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were edited with sequence assembly software ATGC Ver. 6 (Genetyx, Japan) and we used MEGA5. 1 software (Tamura *et al.*, 2011) to align nucleotide sequencing. A haplotype network was drawn using TCS ver. 1.21 (Clement *et al.*, 2000).

Results and Discussion

We identified three haplotypes through the survey. Among the 39 specimens analyzed, the haplotype (Asia-Oceania 1) of 27 individuals was the same as the most frequent one identified in Watabiki *et al.* (2013). One individual had the same haplotype (Asia-Oceania 3) as one specimen noted by Watabiki *et al.* (2013). In addition, we found one more haplotype (Asia-Oceania 2) in 11 individuals, which was identical to one individual from Australia. Therefore, total numbers of each of the three haplotypes included in Watabiki *et al.* (2013) plus those included in this paper were 67 individuals of Asia-

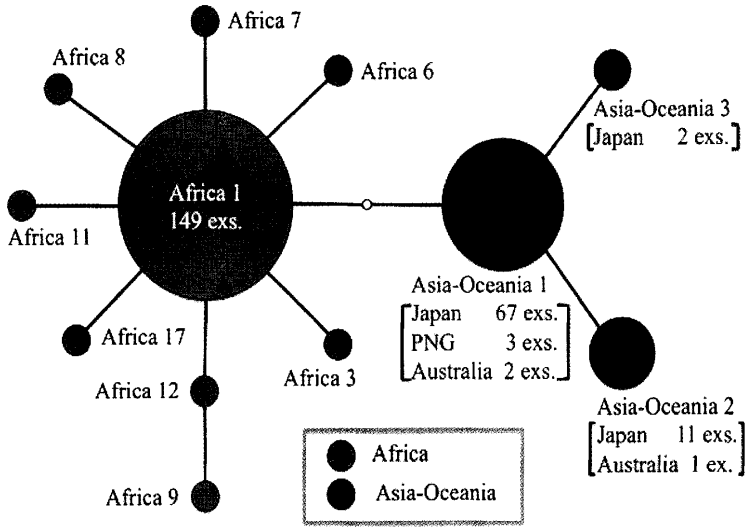

Fig. 2. Haplotype network of African and Asia-Oceanian *Spodoptera exempta* based on 585 bp of the mitochondrial *COI* gene. Numbers of individuals of each haplotypes are shown if not single. The haplotype of Africa 10 became the same as that of Africa 1 because they are compared with Asia-Oceanian haplotypes together. Therefore the haplotype of Africa 10 is not shown and not counted as Africa 1 here. PNG means Papua New Guinea.

Oceania 1, 11 specimens of Asia-Oceania 2 and two examples of Asia-Oceania 3 as shown in Fig. 2.

Although Watabiki *et al.* (2013) showed a neighbor-joining tree for intraspecific haplotypes of African and Japanese *S. exempta* based on the mitochondrial *COI* gene, seven among the 17 African haplotypes to which they referred from Graham and Wilson (2012) were misidentifications, as mentioned by Graham and Wilson (2013). Therefore these seven haplotypes were excluded from our study at this time.

Among the 10 African haplotypes, nine of them are not frequent and are represented by only one individual; in contrast, the three Asia-Oceanian haplotypes are represented by plural examples as shown in the haplotype network (Fig. 2). The haplotype of the specimens from Papua New Guinea (JX970437-JX970439) and Australia (HQ950416, HQ950418) registered in GenBank is identical with that of 67 examples from Japan (Asia-Oceania 1). The haplotype of a sample (HQ950417) from Australia is the same as that of 11 individuals from Japan (Asia-Oceania 2). This result may suggest that they are widely distributed haplotypes from South East Asia, Papua New Guinea to Australia. However, haplotypes from Asia-Oceanian regions have not been analyzed except for those from Japan, Australia and Papua New Guinea. Therefore, a survey of haplotypes from other Asia-Oceanian regions will be necessary. To illustrate this, the recent sequencing of a natural history collection mentioned in Hebert *et al.* (2013) only recently revealed the haplotype data from Australia used in this study. If such data is accumulated in the future, we may be able to draw conclusions about the migratory route of the moths more precisely in the near future.

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摘　要

日本で害虫化したアフリカシロナヨトウ（ヤガ科）のDNAバーコーディング追加情報（吉松慎一・綿引大祐・西岡稔彦・中村浩昭・山口卓宏・嶽崎　研・島谷真幸・上里卓己）

アフリカシロナヨトウ*Spodoptera exempta* (Walker) (Fig. 1)はアフリカでは著名な害虫で，他にもアジアの熱帯地域，オーストラリア，ハワイを含む太平洋の島嶼に分布し，成虫は数百キロメートルという長距離の移動が可能である．雌成虫(Fig. 1: C, D)は前翅がほぼ一様に黒色を呈するのに対し，雄成虫(Fig. 1: A, B)では前翅の地色は黒色であるが，中央部から亜外縁部にかけて淡色部を備えることで区別できる．日本からは，2010年より前には成虫が僅かに得られていたが，南西諸島で2010年夏～秋に初めて害虫化した(吉松ら，2011)．その後，福田（2012）は沖縄で被害が確認された2010年8月とほぼ同時期に鹿児島県本土の薩摩郡紫尾山（しびさん，標高1,067m）で2個体を採集していたことを報告した．2010年には，沖縄県と鹿児島県ではアフリカシロナヨトウの合成性フェロモントラップを用いて調査を実施したが，アフリカシロナヨトウ以外にも同属の複数種が得られていた．そこで，綿引ら（2013）は雄交尾器形態およびDNAバーコーディングにより，本属の種の識別法を開発し，本種のmtDNA（*COI*）に2つのハプロタイプを確認した．

その後，沖縄県で2012年にフェロモントラップで成虫を採集し，鹿児島県の離島で2013年に野外で本種幼虫・蛹を採集した．また，これら沖縄産と鹿児島産の成虫標本の解析の結果，綿引ら（2013）が報告した2種類のハプロタイプとは異なる1種類のハプロタイプを認め，2010年には1個体でしか確認されなかったハプロタイプを今回さらに1個体で認めた．綿引ら（2013）と今回の結果を併せた日本を含むアジア・オセアニアの3個のハプロタイプとGraham and Wilson (2013)で報告されたアフリカ産の10個のハプロタイプをハプロタイプネットワークに示したが（Fig. 2），両者は2つのグループに分けることができ，少なくとも2塩基が異なった．GenBankに登録されていたパプアニューギニア産3個体とオーストラリア産2個体のハプロタイプは日本産の最も頻度の高いハプロタイプ（Asia-Oceania 1）に一致し，また，今回新たに日本から見いだしたハプロタイプはオーストラリア産1個体のハプロタイプ（Asia-Oceania 2）に一致したことから，これらのハプロタイプは東南アジアからニューギニア，オーストラリアにかけて広く分布している可能性があることが示唆された．東南アジア各地での本種のハプロタイプはほとんど解析されておらず，日本で発生した個体群がどこから飛来・侵入してきたのかを現時点で推測することはできない．今後の東南アジアを中心とした地域における本種のハプロタイプの解析を待ちたい．

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